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The Determination of Methionine in Proteins by Gas–Liquid Chromatography

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Intact methionine residues in proteins were rapidly and precisely determined by measuring methyl thiocyanate released during the reaction with CNBr and separated by g.l.c. Conditions for the reaction and for chromatography on columns of Porapak P-S are described. The recovery of methyl thiocyanate from several methionine derivatives and analogues was examined. Carbamoylmethionine was adopted as a stable primary standard and ethyl thiocyanate as internal standard. The measured methionine content of several isolated proteins was close to the theoretical value indicated by previous work and the results for these and a range of food proteins agreed well with results obtained by ion-exchange chromatography after performic acid oxidation. Since CNBr does not react with methionine sulphoxide and a preliminary hydrolysis is not required, the method discriminates between methionine and any methionine sulphoxide that may be present. It could be useful in studies on the nutritional availability of methionine in processed foods.

Since the work of Ray & Koshland (1962) and Njaa (1962*a*), the problems of measuring the methionine, as distinct from the methionine sulphoxide, content of proteins have been widely documented. The acid hydrolysis of proteins before analysis disturbs the original equilibrium between the two compounds so that the composition of the hydrolysate no longer reflects that of the protein. Alkaline hydrolysis has been used as an alternative procedure to establish the degree of oxidation of methionine residues in lysozyme (Jori *et al.*, 1968). In our experience with oxidized casein, the sum of methionine and methionine sulphoxide found after alkaline hydrolysis was less than the amount of methionine sulphone recovered after performic acid oxidation and hydrolysis. At that time, the loss could not be ascribed to either methionine or methionine sulphoxide. Losses of 10–25% during alkaline hydrolysis of free methionine sulphoxide were reported by Neumann *et al.* (1962). A specific chemical test for intact methionine would be useful for monitoring oxidative changes, whether deliberate or accidental. Lysozyme (Jori *et al.*, 1968) and pituitary corticotropin (Dedman *et al.*, 1961) are just two of the growing number of enzymes and hormones that have been shown, by controlled oxidation, to depend on intact methionine residues for their biological activity. The protection of methionine residues during peptide synthesis has been achieved by incorporating residues of methionine sulphoxide which are reduced to methionine on completion of the synthesis (Iselin, 1961; Hoffmann *et al.*, 1969). The accidental oxidation of some methionine residues has explained the heterogeneity observed in preparations of calcitonin (Brewer *et al.*, 1968) and in ribonuclease that has been subjected to electrofocusing in the absence of a reducing agent (Jacobs, 1971). The extent to which methionine sulphoxide may nutritionally replace methionine in food proteins, and the importance of modifications at methionine residues damaged during processing and storage, can be assessed only by an analytical method that discriminates between the two compounds.

The reaction of CNBr with peptide-bound methionine is highly specific (Gross & Witkop, 1962); methionine sulphoxide does not react. Schroeder *et al.* (1969) found homoserine formation from methionine residues in catalase to be almost quantitative, although subsequent peptide cleavage may be hindered by adjacent serine or threonine residues. Inglis & Edman (1970) and Ellinger & Smith (1971) indicated that MeSCN (methyl thiocyanate), which is released as homoserine is formed, may be determined by g.l.c. and can serve as a measure of the methionine content of protein without the need for hydrolysis. The present paper gives an account of the conditions that were developed to use g.l.c. for the quantitative determination of intact methionine in proteins.

Materials and Methods

Materials

CNBr, MeSCN and EtSCN (ethyl thiocyanate) were purchased from Eastman Kodak Co., Rochester, NY, U.S.A. The thiocyanates were dried over 3mm pellets of molecular sieve (Type 4A; BDH, Poole, Dorset, U.K.). Formic acid (99–100%) was a gift from BASF United Kingdom Ltd., London SW7 1SA, U.K. 'Merck' 98–100% formic acid (Pro analysi grade) was supplied through Anderman and Co.,

London SE1 2RA, U.K. L-Methionine, DL-methionine, L-methionine-DL-sulphoxide, *N*-acetyl-DL-methionine, L-methionyl-L-alanine, DL-alanyl-DL-methionine, *N*-carbamoyl-DL-methionine, *S*-methyl-L-cysteine and bovine pancreatic ribonuclease (type IIA) were supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Hen's egg ovalbumin was given by Dr. J. Conchie from this laboratory, and pig pepsinogen D by Dr. A. P. Ryle (Department of Biochemistry, University of Edinburgh, U.K.). Lysozyme (crystalline) and bovine plasma albumin were purchased from Armour Laboratories, Eastbourne, Sussex, U.K., and 'Pentex' β-lactoglobulin (crystalline) was from Miles–Seravac, Stoke Poges, Bucks. SL2 4LY, U.K. Dimethyldichlorosilane was obtained from Hopkin and Williams Ltd., Romford, Essex RM1 1HA, U.K.

Porapak P-S (80–100mesh) from batches 973 and 1042 was obtained through Phase Separations Ltd., Queensferry, Flints., Wales, U.K. Batches 1119 and 1110 were gifts from Waters Associates Instruments Ltd., Heavesley, Cheshire SK2 6PT, U.K.

Carbamoylmethionine was prepared as follows. DL-Methionine (10g) dispersed in 75ml of water was heated on a boiling-water bath and solid KCNO (7g) was added. Once pH7 was reached, neutrality was maintained by the addition of 1M-HCl, and heating was continued for 90min. Conc. HCl was added to the cooled solution until pH3 was reached. Crystallization in ice, and filtration, were followed by three recrystallizations from methanol. The m.p. of carbamoyl-DL-methionine stabilized at 140°C, and that of carbamoyl-L-methionine, similarly prepared, at 155°C.

Methods

Preparation of samples for g.l.c. Samples containing not less than 2μmol of methionine were allowed to react with 65mg of CNBr overnight at 23°C in 3ml of 70% (v/v) formic acid. Feeding-stuffs were ground to pass a 60-mesh sieve and the dry samples were well dispersed in 1ml of conc. formic acid before dilution with 1ml of 40% (v/v) formic acid and the addition of CNBr in solution in 1ml of 70% formic acid. Standard solutions of MeSCN and EtSCN were prepared in 40% formic acid. The carbamoylmethionine standard was an aqueous solution. After 17h, residual CNBr was destroyed with a slight excess of $Na_2S_2O_3$ contained in 1ml of water. The required concentration of $Na_2S_2O_3$ was established by titration of CNBr by using 5% (v/v) *o*-tolidine in pyridine (Feigl, 1966) as external indicator. Back-titration with iodine would give a misleading end-point because one of the reaction products is Na_2SO_3. An internal standard (1ml of EtSCN) was dispensed with the utmost precision from a Warburg pipette. All other additions were made from a 1.0ml Eppendorf pipette, by taking the precautions advocated by Ellis (1973). The sample size and pipetted volumes may be decreased to one-tenth with only slight loss of precision. The reaction mixture was centrifuged at approx. 2000*g* for 20min before application of the supernatant solution to the g.l.c. column.

G.l.c. of methyl thiocyanate. A Pye 104 gas chromatograph with flame-ionization detector was used throughout. All gases, argon as carrier gas at 30ml/min, hydrogen at 30ml/min and air at approx. 500ml/min, were passed through molecular sieve (type 13X; Pye Unicam Ltd., Cambridge CB1 2PX, U.K.). The glass column (0.64cm internal diam., 91.4cm long) and glass-wool plugs were silanated with three separate portions of 5% (v/v) dimethyldichlorosilane in toluene followed by washing with methanol until free from HCl. Porapak P-S was filled in short segments into the columns by tapping at the same time as suction from a vacuum pump was applied. A small excess of Porapak P-S was added to allow for shrinkage during conditioning. This was normally done in a stream of argon at 15ml/min for 15min at 50°C, then at a temperature rising at 2°C/min to 195°C and eventually overnight at 195–200°C. Occasionally when a batch of Porapak P-S gave abnormal resolutions this was remedied by preconditioning the packing in a stream of air as described by Goodfellow & Webber (1972). During the analysis, oven and detector temperatures were kept at 140 and 200°C respectively. The detector output was attenuated at 2×10^{-11}A.

Portions of the reaction mixture were applied to the top of the column with a 1μl syringe (type 1B, with interchangeable plunger and 11.5cm needle; Scientific Glass Engineering Pty. Ltd., London NW2 7AY, U.K.). Syringes of this type well withstood contact with formic acid and, in the event of corrosion, parts can easily be replaced. The output from the flame-ionization detector was recorded on a Honeywell recorder (1mV range full-scale deflexion). The peak areas were calculated from the product of peak height and width at half-height. MeSCN and EtSCN had retention times of 11 and 18.5min respectively.

Results were calculated as described by Kuksis (1966). To ascertain continuity of conditions, the constant that is derived from the ratio of areas from MeSCN standard and the internal EtSCN standard was re-established daily from quadruplicate injections. As a rule, the result for each CNBr reaction mixture was calculated by using the mean area ratio from three injections.

Results and Discussion

Strictly quantitative evaluations by g.l.c. require the injection of an internal standard together with

samples and standard solutions. Addison & Ackman (1968) stressed the need for an internal standard that is closely related to the test material, to compensate for both chemical and physical losses that may be incurred. EtSCN was accordingly chosen as the internal standard. The occurrence of ethionine and *S*-ethylcysteine is rare, and interference through EtSCN that could be derived from these sources can generally be ignored.

Originally 5% Carbowax on 20 M Chromosorb W was prepared and used as column packing (Ellinger & Smith, 1971). Coating the solid phase to give reproducible performance with each batch proved difficult, and the new internal standard was not adequately resolved. Beads of Porapak P-S were found to be a more effective column packing. Separations are achieved directly on the surface of this porous ethylvinylbenzene–styrene–divinylbenzene polymer; in the absence of a film of liquid phase, the surface area for a given weight of polymer is large, and good resolution is obtained on short columns. Fig. 1 illustrates the resolution of MeSCN and EtSCN on Porapak P-S. There was no evidence of losses through adsorption. Since the beads are physically robust, failing performance of columns after prolonged used could be restored repeatedly by replacing the 5 cm top layer of packing that contained charred residues from injected samples.

The direct chromatography of the CNBr reaction mixture on columns of Porapak P-S presented two problems. First, CNBr reacts with the double bonds of aromatic compounds. The excess of reagent from a single injection reacted with the polymer packing and completely destroyed column performance. A small excess of $Na_2S_2O_3$ was therefore added to eliminate residual CNBr before injection of the reaction mixture. Secondly, the effectiveness of Porapak P-S was determined by the quality of the formic acid that serves as solvent. Acetic acid that is generally present as an impurity in formic acid ionizes in the detector and the peak, with a tendency to tail, emerges just ahead of MeSCN. Under the present conditions, formic acid that originated from a general cracking process and contained about 0.7% acetic acid impaired the resolution of MeSCN and hence the precision of peak-area measurements. Attempts to decrease the acetic acid contamination were unsuccessful. When formic acid from a more specific synthetic process, containing about 0.02% acetic acid, was used (as in the samples listed under 'Materials'), the problem ceased to exist.

As a test of the g.l.c. conditions, standard solutions of MeSCN and EtSCN were injected directly on to the columns. Fig. 2 illustrates the linear response to injections of increasing concentrations of MeSCN in the presence of constant EtSCN. Similarly, a linear response to increasing concentrations of EtSCN and constant MeSCN was obtained. The steeper slope reflects the greater ionization per mol arising from the additional methylene group in EtSCN.

Methionine was next used as standard to test the combined CNBr reaction and g.l.c. of the MeSCN formed. The dose/response plot was again linear (Fig. 2), but the improved precision of the method revealed that recoveries were not 100%. Even when

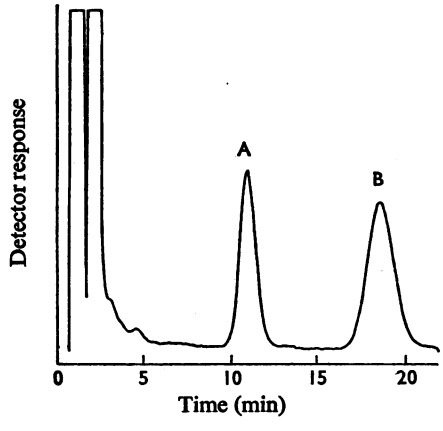

Fig. 1. *Gas–liquid chromatogram of (A) MeSCN and (B) EtSCN*

Chromatographic conditions are described under 'Methods'.

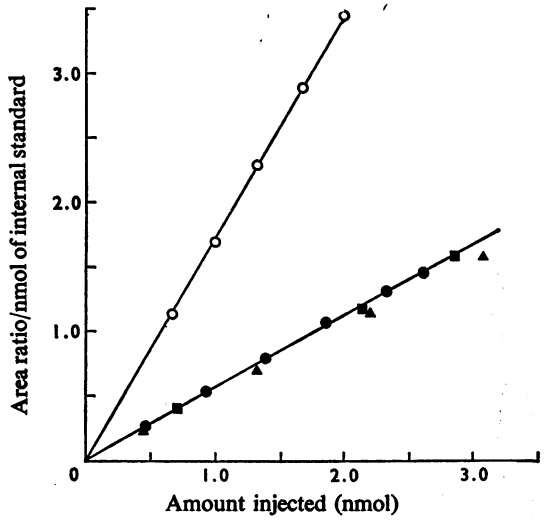

Fig. 2. *G.l.c. dose/response curves from standard solutions of (●) MeSCN and (○) EtSCN directly and of (▲) methionine and (■) carbamoylmethionine derived by reaction with CNBr*

Conditions of reaction are described under 'Methods'.

Table 1. *Recovery of MeSCN from methionine, methionine derivatives and analogues*

Conditions for MeSCN formation and its determination by g.l.c. are described under 'Methods'.

	Recovery (%)
Methionine sulphoxide	0
Methionine *S*-methylsulphonium chloride	0
Methionine hydantoin	0
S-Methylcysteine	0
Met-Ala	54
Carbamoyl-*S*-methylcysteine	81
Methionine	90
Ala-Met	94
N-Acetylmethionine	96
Carbamoylmethionine	100

the reaction conditions were varied and the reaction time was extended, the theoretical yield of MeSCN was not obtained from free methionine. A similarly limited release of [^{14}C]methyl thiocyanate from [*Me*-^{14}C]methionine was observed by Clark *et al.* (1974). The slow reaction rate of free methionine had previously been reported (Inglis & Edman, 1970; Ellinger & Smith, 1971). Gross (1967) had found that, under acid condition, CNBr slowly oxidizes cysteine to cysteic acid. A similar oxidation of methionine to methionine sulphoxide, which then no longer reacts, may be the result of the slow reaction rate of methionine and its prolonged exposure to the reagent. This possibility was supported by ion-exchange chromatography of the reaction mixture after 17h. The methionine and methionine sulphoxide recovered after treatment with $Na_2S_2O_3$, evaporation and taking up in 0.2M-sodium citrate buffer, pH2.0, accounted for nearly 10% of the initial methionine. Free methionine is therefore unsuitable as standard for this determination of methionine in proteins, and samples containing appreciable amounts of free methionine must be expected to yield slightly low results.

Table 1 lists the recoveries that were obtained from several methionine derivatives and from some analogues of methionine. High recoveries were paralleled by fast reaction rates (Fig. 3). These results confirm the favourable affect of the free carboxyl group (Gross, 1967; Inglis & Edman, 1970), and further indicate that the nature of the substitution at the amino group that hinders when free can have an additional effect. Carbamoylmethionine proved to be the most rapidly reacting of the methionine derivatives; its linear calibration curve (Fig. 2) completely overlapped that of MeSCN, and the quantitative recoveries were highly reproducible. It is tempting to regard the release of MeSCN as an index of the co-ordinated lactone formation. Steric hindrance to

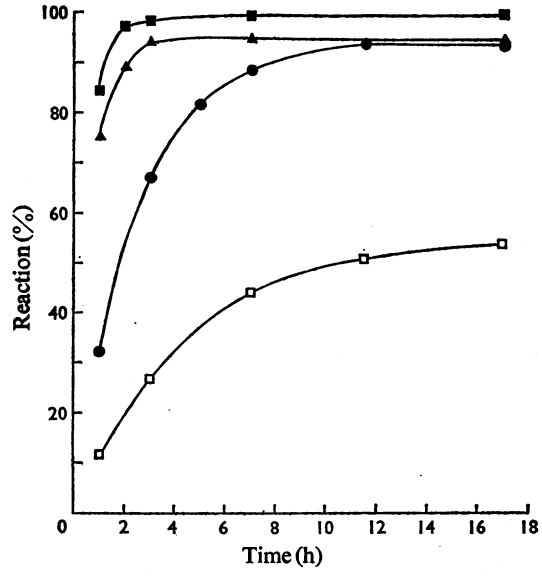

Fig. 3. *CNBr reaction rates with* (●) *methionine*, (□) *Met-Ala*, (▲) *Ala-Met and* (■) *carbamoylmethionine*

Conditions of reaction are described under 'Methods'.

lactone formation could account for the lack of reaction of methionine hydantoin. Similarly, free *S*-methylcysteine failed to release MeSCN because the four-membered lactone ring of serine does not form readily. However, for peptide-bound *S*-methylcysteine, Awad & Wilcox (1964) postulated an alternative reaction with CNBr, namely the formation of an oxazoline ring involving the carbonyl group on the amino side of *S*-methylcysteine. The extensive release of MeSCN from carbamoyl-*S*-methylcysteine supports this explanation. MeSCN may be cleaved from peptide-bound *S*-methylcysteine by yet another mechanism. The formation of a dehydroalanine residue after β-elimination was reported to predominate at higher reaction temperatures (Gross *et al.*, 1967). This accounted for recoveries of 20% and 97% from *S*-methylcysteine and carbamoyl-*S*-methylcysteine respectively when the reaction temperature was raised to 50°C, whereas the recovery from methionine remained unaltered.

With carbamoylmethionine as the standard, the methionine content of several purified proteins was measured. Table 2 relates the results obtained by g.l.c. to values obtained by ion-exchange chromatography after performic acid oxidation and to theoretical published values. The molecular weights that were used to express results in terms of residues/mol are also listed. For lysozyme, pepsinogen D and ovalbumin, the results from g.l.c. were close to

Table 2. *Recovery of methionine from isolated proteins*

Values are the means ± S.E.M. for the numbers of determinations given in parentheses.

	Recovery (residues/mol)				
	G.l.c. after CNBr reaction	Ion exchange after performic acid oxidation	Theoretical	Mol.wt.	Reference
Lysozyme	1.89 ± 0.01 (4)	1.68 ± 0.03 (5)	2	14307	Canfield (1963)
Pepsinogen D	3.94 ± 0.03 (5)	3.88 ± 0.12 (3)	4	38944	Rajagopalan *et al.* (1966)
β-Lactoglobulin AB	3.72 ± 0.02 (4)	3.52 ± 0.06 (4)	4	18384	Braunitzer *et al.* (1973)
Ovalbumin	14.39 ± 0.08 (6)	14.45 ± 0.34 (4)	15	45000	Marshall & Neuberger (1972); Smith & Back (1970)
Bovine plasma albumin	3.69 ± 0.04 (4)	3.95 ± 0.09 (6)	4	65500	King & Spencer (1970)
Bovine ribonuclease	3.37 ± 0.02 (6)	3.54 ± 0.02 (3)	4	13683	Hirs *et al.* (1956)

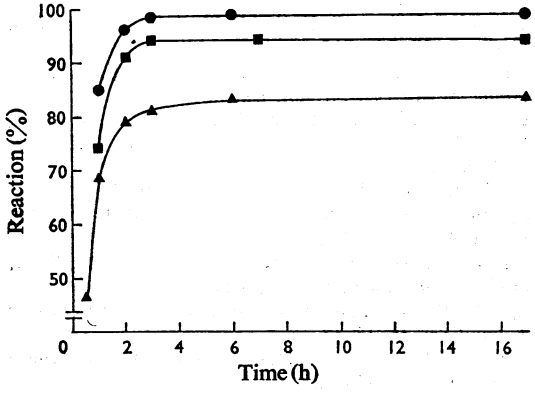

Fig. 4. *CNBr reaction rates with* (●) *pepsinogen,* (■) *bovine plasma albumin and* (▲) *ribonuclease*

Conditions of reaction are described under 'Methods'.

Table 3. *Methionine content of some food proteins*

Values are the means ± S.E.M. for the numbers of determinations given in parentheses.

	Methionine content (g/16g of N)	
	G.l.c. after CNBr reaction	Ion exchange after performic acid oxidation
Casein	3.14 ± 0.02 (4)	2.84 ± 0.03 (4)
Whitefish meal	2.78 ± 0.02 (4)	2.83 ± 0.04 (4)
Pilchard meal	1.92 ± 0.02 (4)	2.60 ± 0.04 (4)
Stabilized pilchard meal	2.40 ± 0.01 (4)	2.50 ± 0.03 (4)
Freeze-dried lucerne leaf protein	1.98 ± 0.01 (4)	2.14 ± 0.05 (4)
Soya-bean meal	1.35 ± 0.01 (4)	1.38 ± 0.04 (4)
Wheat gluten	1.48 ± 0.00 (4)	1.52 ± 0.02 (3)
Hydrocarbon-grown yeast	1.41 ± 0.01 (5)	1.72 ± 0.01 (6)

theoretical. The relatively low recovery from bovine plasma albumin suggested the presence of some methionine sulphoxide in the sample, which had been stored for several years. In view of the normally rapid reaction rate (Fig. 4). it seems unlikely that the low value arose for structural reasons. With ribonuclease such a hindrance could well be expected. The relatively low reactivity of one of the methionine residues in ribonuclease has long been established (Neumann *et al.*, 1962). The unusual Met-Met sequence at residues 29 and 30 in particular has made cleavage by the CNBr reaction with ribonuclease a special case. With 0.1 M-HCl as the reaction medium, Link & Stark (1968) were unable to increase CNBr cleavage at residue 30 above 33% of the theoretical value. They attributed the poor cleavage at residue 30 to a very rapid reaction by residue 29 leaving residue 30 in the *N*-terminal position very much less reactive towards CNBr. The low recovery of MeSCN from Met-Ala (Table 1) appears to justify this explanation. But for Met-Ala the low recovery was paralleled by a marked fall in the reaction rate (Fig. 3). The methionine content of ribonuclease, which was low when determined after performic acid oxidation, was slightly lower when determined by g.l.c. But the reaction rate was rapid and hardly distinguishable from that of pepsinogen (Fig. 4). If the release of MeSCN from residue 30 were indeed hindered, the effect on the rate of release must be obscured because only one of four methionine residues is affected. Gross & Witkop (1962) advocated 0.1 M-HCl as the solvent necessary for maximum reaction between CNBr and ribonuclease. The change to reaction in 0.1 M-HCl, instead of 70% formic acid, did not alter the g.l.c. recovery of methionine.

The two methods of methionine determination were similarly applied to several food proteins (Table 3). Agreement between the two methods was generally good. The difference that was

observed for the unstabilized pilchard meal indicates that peroxidation of the lipids present caused some oxidation to occur at methionine residues and that this oxidation could be prevented by antioxidants. The result for the sample of hydrocarbon-grown yeast confirmed the observation made in an earlier feeding trial with pigs, which showed that the methionine determined as the sulphone after performic acid oxidation had not been fully utilized (G. M. Ellinger, unpublished work). Problems were encountered with the determination of methionine in cereals and in the seeds of legumes. During the overnight reaction, cereal starches swell and gel in the 70% formic acid suspension. Many varieties of beans contain substantial amounts of γ-glutamyl-*S*-methylcysteine, which also yields MeSCN with CNBr.

Several workers (Lea & Hannan, 1950; Carpenter *et al.*, 1957; Miller *et al.*, 1965) have been concerned with the loss of nutritional availability of methionine in food proteins after processing and storage. The nature of the change that causes methionine residues in protein to become nutritionally unavailable, while remaining convertible into methionine sulphone in the standard analytical procedure (Moore, 1963), is not known. The oxidation to methionine sulphoxide had been considered to be responsible, but the nutritional role of methionine sulphoxide remains controversial. As a supplement to a soya-bean diet, free methionine sulphoxide derived from L-methionine could replace methionine in short-term growth tests with weanling rats (Njaa, 1962*b*). Free methionine sulphoxide had restricted value for growing rats fed on synthetic diets from which methionine had been altogether omitted (Miller *et al.*, 1970). In modified food proteins, problems would arise primarily from the presence of peptide-bound rather than free methionine sulphoxide. Ellinger & Palmer (1969) converted methionine residues in casein into methionine sulphoxide by treatment with H_2O_2 under acid conditions; no methionine sulphone could be detected. Both the net protein utilization by rats, and the response by *Streptococcus zymogenes* (Ford, 1962) were decreased, although methionine sulphoxide had been nearly completely utilized in the latter assay. Cuq *et al.* (1973) examined the release of amino acids during the digestion *in vitro* of casein similarly oxidized by increasing concentrations of H_2O_2. Although the treatments had no effect on the appearance of free amino acids generally, as the content of bound methionine sulphoxide increased the release of methionine decreased; no free methionine sulphoxide was found in the digest.

It appears that although free methionine sulphoxide may be reduced in the body, the peptide-bound form is less readily released by the digestive enzymes than is bound methionine. Preliminary enzymic digestion has been the basis of both Ford's (1962) microbiological assay for available methionine and the recent measurement by sodium nitroprusside (Pieniążek *et al.*, 1975). The present highly specific determination, which requires no preliminary hydrolysis, could be a useful tool in the study of nutritional availability of methionine in food proteins.

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